

Reduced Activity of Antioxidant Machinery Is Correlated with Suppression of Totipotency in Plant Protoplasts¹

Anastasia K. Papadakis, Charalambos I. Siminis², and Kalliopi A. Roubelakis-Angelakis*

Department of Biology, University of Crete, P.O. Box 2208, 71409 Heraklio, Greece

We previously showed that during protoplast isolation, an oxidative burst occurred and the generation of active oxygen species was differentially mediated in tobacco (*Nicotiana tabacum*) and grapevine (*Vitis vinifera*), accompanied by significant quantitative differences (A.K. Papadakis, K.A. Roubelakis-Angelakis [1999] *Plant Physiol* 127: 197–205). We have now further tested if the expression of totipotency in protoplasts is related to the activity of cellular antioxidant machinery during protoplast culture. Totipotent (T) tobacco protoplasts had 2-fold lower contents of intracellular $O_2^{\cdot-}$ and H_2O_2 and 7-fold lower levels of $O_2^{\cdot-}$ and H_2O_2 in the culture medium, compared with non-totipotent (NT) tobacco protoplasts. Addition of alkaline dimethylsulfoxide, known to generate $O_2^{\cdot-}$, resulted in isolation of tobacco protoplasts with reduced viability and cell division potential during subsequent culture. Active oxygen species levels decreased in tobacco and grapevine protoplasts during culturing, although higher contents of $O_2^{\cdot-}$ and H_2O_2 were still found in NT- compared with T-tobacco protoplasts, after 8 d in culture. In T-tobacco protoplasts, the reduced forms of ascorbate and glutathione predominated, whereas in NT-tobacco and grapevine protoplasts, the oxidized forms predominated. In addition, T-tobacco protoplasts exhibited severalfold lower lipid peroxidation than NT-tobacco and grapevine protoplasts. Furthermore, several antioxidant enzyme activities were increased in T-tobacco protoplasts. Superoxide dismutase activity increased in tobacco, but not in grapevine protoplasts during culturing due to the increased expression of cytoplasmic Cu/Zn-superoxide dismutase. The increase was only sustained in T-tobacco protoplasts for d 8. Together, these results suggest that suppressed expression of totipotency in protoplasts is correlated with reduced activity of the cellular antioxidant machinery.

Plant protoplasts, i.e. plant cells devoid of cell walls, represent a very efficient experimental model and are also valuable tools for biotechnological applications, such as somatic hybridization and genetic transformation, providing they exhibit totipotency, i.e. can regenerate to produce whole plants. However, protoplasts from most of the agriculturally important plant species exhibit recalcitrance to regeneration. The reasons why these protoplasts do not express totipotency are largely unknown. They could be as simple as deficiencies in the culture medium or much more complex, such that totipotency through induction of high frequency cell divisions and morphogenic expression requires the transduction and perception of specific signals and gene expression, which are blocked in non-totipotent (NT) protoplasts. Grapevine (*Vitis vinifera*) is a perennial woody plant species whose protoplasts exhibit recalcitrance to regeneration, in contrast to tobacco (*Nicotiana tabacum*) protoplasts, which are readily totipotent (T) or NT depending upon the method of preparation.

Oxidative stress, resulting from imbalance between active oxygen species (AOS) generation and

antioxidant capacity of cells, has been proposed to contribute to recalcitrance of plant protoplasts (Cutler et al., 1991; Roubelakis-Angelakis, 1993). AOS such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH$) can accumulate in response to biotic and abiotic stress (Scandalios, 1993; Foyer et al., 1997). They can have a detrimental effect on the metabolism, growth, and development of cells through their ability to initiate reaction cascades that result in the production of toxic chemical species, such as hydroxyl radicals and lipid peroxides, ending in cell dysfunction and death (Bowler et al., 1992; Scandalios, 1993; Alscher et al., 1997). In parallel, however, AOS may also have a positive role in plant growth and development; H_2O_2 participates in the peroxidase-mediated intramolecular isotyrosine ether cross-linking of reconstituted cell walls (Iiyama et al., 1994) and $O_2^{\cdot-}$ and H_2O_2 may serve as signal molecules, stimulating defense responses (Jabs et al., 1997; Lamb and Dixon, 1997). A dual role for H_2O_2 in the regeneration of protoplasts has also been shown (de Marco and Roubelakis-Angelakis, 1996a, 1996b).

Living organisms have developed a wide range of antioxidant strategies for protection from oxidative damage, based on direct radical scavenging ability of certain chemical species such as ascorbate, glutathione, α -tocopherol, and polyamines (Larson, 1988; Noctor and Foyer, 1998), and also enzymic reactions

¹ This work was supported by the Interreg II Project.

² Present address: Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece.

* Corresponding author; e-mail poproube@biology.uoc.gr; fax 30–81–394459.

(Scandalios, 1993; Foyer et al., 1994). Superoxide dismutase (SOD) is the first enzyme in the detoxifying process (Scandalios, 1993); it disproportionates $O_2^{\cdot-}$ to H_2O_2 , which is further reduced to water by catalase in peroxisomes and by ascorbate peroxidase (APO) in chloroplasts and the cytosol (Foyer and Halliwell, 1976; Asada, 1992; Alscher et al., 1997). Oxidized ascorbate resulting from APO activity is reduced by monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) in reactions, which exploit NADPH and glutathione; glutathione reductase (GR) completes the cycle maintaining a high ratio between reduced glutathione (GSH) and its oxidized form (GSSG) in the cell (Alscher et al., 1997). Moreover, GSH is also a substrate for glutathione peroxidase (GS-POX), which has a preference for organic hydroperoxides both in animals and plants (Eshdat et al., 1997).

Results from previous comparative work using tobacco and grapevine protoplasts have shown significant differences in their oxidative and antioxidative machinery (Siminis et al., 1993, 1994; de Marco and Roubelakis-Angelakis, 1996a, 1996b, 1997, 1999; Papadakis and Roubelakis-Angelakis, 1999). Establishment of a protocol for isolating T- and NT-tobacco protoplasts (Siminis et al., 1994) enabled their comparison. Total POX activity was significantly higher in T-tobacco than in grapevine protoplasts during culturing, with expression of specific isoenzymes; during the first days in the culture, the cell wall-bound acidic isoenzymes, whereas later in the culture, the basic isoenzymes were induced (Siminis et al., 1993). Also, the H_2O_2 -scavenging activity of catalase was increased in grapevine but not in tobacco protoplasts during culturing; in NT-tobacco and grapevine protoplasts the most catalatic isoenzymes predominated, whereas in T-tobacco protoplasts, the de novo accumulation of the catalase β -subunit gave rise to the less catalatic, acidic isoenzymes (Siminis et al., 1994). APO activity and tran-

script of cytoplasmic isoform were present only in T-tobacco protoplasts (de Marco and Roubelakis-Angelakis, 1996b) and the activities of antioxidant enzymes of the Halliwell-Asada pathway were significantly lower in non-regenerating tobacco protoplasts (de Marco and Roubelakis-Angelakis, 1996a, 1999). Furthermore, in an effort to identify the mechanisms of AOS generation during protoplast isolation, the contribution of maceration enzymes and wounding of leaf tissue were studied (Papadakis and Roubelakis-Angelakis, 1999). Use of the non-purified cellulase Onozuka (Yakult Honsha Co., Tokyo), which is known to contain xylanase and possibly other enzymic contaminants (Fuchs et al., 1989), induced a burst of $O_2^{\cdot-}$ and H_2O_2 accumulation in tobacco leaf strips, whereas significantly lower levels of both AOS were accumulated in treated grapevine leaf strips. Wounding alone or use of a purified cellulase (Worthington Biochemical Corporation, Freehold, NJ) did not elicit AOS production. Superoxides and H_2O_2 were shown to be generated by different enzymic systems in the two plant species; in tobacco, two different AOS synthase activities were revealed: a mammalian-like NADPH oxidase and an NAD(P)H oxidase-peroxidase. In grapevine only the latter activity was detected.

In this work, we have attempted to correlate the totipotency of protoplasts with AOS accumulation, intracellularly and extracellularly in the culture medium, during an 8-d culture period. Also, we have monitored lipid peroxidation, the endogenous levels of reduced and oxidized ascorbate and glutathione and the activities of SOD, APO, MDHAR, DHAR, GR, and GS-POX in cultured T- and NT-tobacco and in grapevine protoplasts. Our results provide further evidence for the correlation of suppressed expression of totipotency with reduced activity of the cellular antioxidant machinery.

Table 1. Accumulation of AOS in freshly isolated protoplasts

Protoplasts were isolated with purified cellulase after 4-h treatment (4–) or with non-purified cellulase after 4- or 16-h treatment (4+ and 16+). AOS were measured in 10^5 protoplasts (intracellularly) and in the respective culture medium (extracellularly). Values are the means \pm SE of 10 independent experiments.

Hours of Maceration	AOS Accumulation			
	Tobacco		Grapevine	
	$O_2^{\cdot-}$	H_2O_2	$O_2^{\cdot-}$	H_2O_2
<i>nM</i>				
Intracellular				
4–	2.2 \pm 0.2	33.9 \pm 1.2	0.7 \pm 0.1	3.6 \pm 0.7
4+	3.9 \pm 0.2	47.3 \pm 2.8	0.8 \pm 0.1	13.3 \pm 1.7
16+	4.2 \pm 0.2	70.8 \pm 3.3	1.6 \pm 0.2	13.4 \pm 1.1
Extracellular				
4–	0.3 \pm 0.0	6.6 \pm 0.7	0.1 \pm 0.0	2.5 \pm 0.1
4+	0.6 \pm 0.1	11.3 \pm 1.2	0.4 \pm 0.1	3.4 \pm 0.2
16+	2.1 \pm 0.1	41.5 \pm 4.9	0.4 \pm 0.1	7.8 \pm 0.3

RESULTS

Effect of the Quality of Maceration Enzymes and of the Length of Maceration Period on AOS Accumulation and on Protoplast Viability and Plating Efficiency

Freshly isolated protoplasts with purified cellulase accumulated $O_2^{\cdot-}$ and H_2O_2 intracellularly and extracellularly, in the culture medium (Table I). More specifically, tobacco and grapevine protoplasts isolated with purified cellulase contained, intracellularly, 88% and 89% of total $O_2^{\cdot-}$ and 84% and 59% of total H_2O_2 , respectively. Use of purified cellulase for 4 h resulted in protoplasts with the highest viability (80.5% and 74.4% viable protoplasts, respectively, for tobacco and grapevine; Fig. 1a) and plating efficiency during subsequent culture (the percentage of viable protoplasts, which had already divided after 8 d in culture, was 74.5% and 1.9%, respectively, for tobacco and grapevine; Fig. 1b). Tobacco protoplasts isolated after a 4-h maceration period with non-purified cellulase, known to result in AOS generation (Ishii, 1987; Papadakis and Roubelakis-Angelakis, 1999), contained, intracellularly, 87% of total $O_2^{\cdot-}$ and 81% of total H_2O_2 ; protoplasts isolated following a 16-h maceration period, contained, intracellularly,

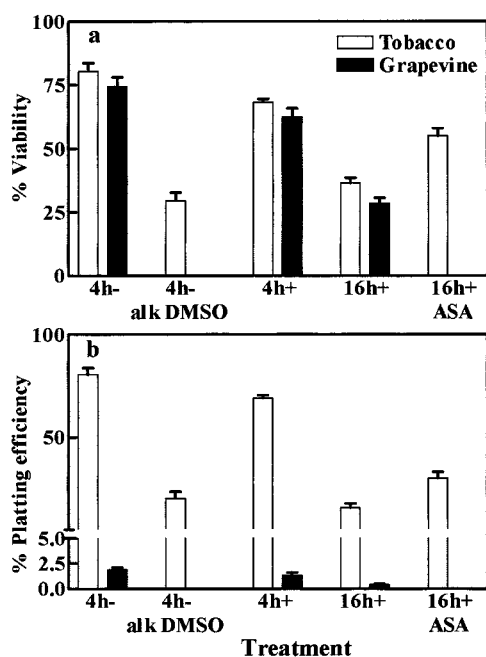


Figure 1. Viability (a) and plating efficiency (b) of tobacco and grapevine mesophyll protoplast after 8 d in culture. Protoplasts were isolated after 4-h maceration with purified cellulase (4 h-), with or without addition of 2.5% (w/v) alkaline dimethylsulfoxide (DMSO), after 4- and 16-h maceration with non-purified cellulase (4 h+ and 16 h+) or after 16-h maceration with non-purified cellulase with the addition of 0.5 mM ascorbate (16 h+, ASA). Viability is expressed as percentage of the initial number of protoplasts; plating efficiency refers to the percentage of viable protoplasts that have divided on the 8th d in culture. Values are the means \pm SE of 10 independent experiments.

66% of total $O_2^{\cdot-}$ and 63% of total H_2O_2 (Table I). The respective values for grapevine protoplasts were 66% and 80% after 4-h maceration and 80% and 63% after 16-h maceration.

Use of non-purified cellulase for 4-h maceration, compared with the purified cellulase, resulted in increased levels of intracellular (+77%) and extracellular (+100%) $O_2^{\cdot-}$ and intracellular (+40%) and extracellular (+71%) H_2O_2 in tobacco protoplasts (Table I). Also, in grapevine protoplasts, the $O_2^{\cdot-}$ level increased intracellularly (+14%) and extracellularly (+300%), and the H_2O_2 level increased intracellularly (+269%) and extracellularly (+36%). The increases in AOS were accompanied by a decrease in viability and plating efficiency of the resultant tobacco protoplasts, but they still could regenerate quite readily (T-tobacco protoplasts, Fig. 1). However, when the maceration procedure was extended to 16 h, the resultant tobacco protoplasts lost their totipotency (NT-tobacco protoplasts). In those NT-tobacco protoplasts, the intracellular $O_2^{\cdot-}$ and H_2O_2 had increased by 8% and 58%, respectively, compared with freshly isolated tobacco protoplasts with 4-h maceration and non-purified cellulase, whereas the extracellular increase was even greater (Table I). Comparable increase in AOS accumulation was also detected in grapevine protoplasts, but they did not regenerate regardless of the quality of maceration enzymes or the duration of the maceration period; by 8 d, they exhibited a negligible number of divisions in all treatments (Fig. 1b), even after 2 weeks in culture (data not shown).

In T-tobacco protoplasts, intracellular and extracellular AOS decreased during the 8-d culture period (Fig. 2). In NT-protoplasts, both intracellular $O_2^{\cdot-}$ and H_2O_2 decreased by d 2 to the same level as in T-protoplasts but then increased again by d 8. Both the extracellular $O_2^{\cdot-}$ and H_2O_2 levels were greater than those in T-tobacco protoplasts and decreased during culturing, with the exception of a minor increase in $O_2^{\cdot-}$ by d 8. In grapevine protoplasts the intracellular and extracellular $O_2^{\cdot-}$ levels decreased during culture, whereas the intracellular and extracellular H_2O_2 remained at the same level as in freshly isolated protoplasts until d 2, after which it decreased, increasing again by d 8 in the case of extracellular H_2O_2 .

To test the effect of AOS on the behavior of T-protoplasts, we used air-saturated DMSO supplemented with 5 mM NaOH, which is known to produce $O_2^{\cdot-}$ (Hyland et al., 1983) at a stable rate for 24 h (data not shown). Addition of 2.5% (v/v) alkaline DMSO to the maceration medium containing purified cellulase for 4 h resulted in tobacco protoplasts with a viability and plating efficiency similar to those of NT-protoplasts isolated after 16-h maceration with non-purified cellulase (Fig. 1). On the other hand, when 0.5 mM ascorbate was added in the maceration medium for NT-tobacco protoplast isolation, the vi-

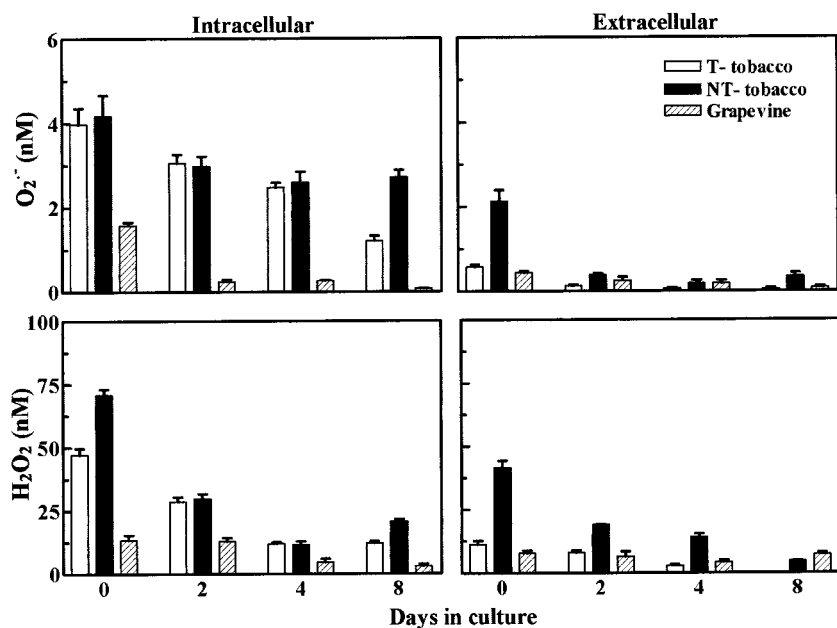


Figure 2. AOS accumulation during protoplast culture. AOS were measured in 10^5 protoplasts (intracellularly) and in the respective culture medium (extracellularly). T- and NT- designates totipotent and non-totipotent tobacco protoplasts. Values are the means \pm SE of 10 independent experiments.

ability and the plating efficiency of these protoplasts significantly increased (Fig. 1).

Endogenous Content and Redox State of Ascorbate and Glutathione in Leaves and Cultured Protoplasts

Grapevine leaf tissue contained 1.5-fold more ASA compared with tobacco leaf tissue (Fig. 3). In both, the reduced form of ascorbate was present in greater amounts than the oxidized form (dehydroascorbate [DHA]). Total ascorbate was 3-fold lower in freshly isolated T-tobacco protoplasts (Fig. 3a) and 4-fold lower in NT-tobacco protoplasts (Fig. 3b) than in

donor leaf tissue. For grapevine protoplasts, total ascorbate was 1.5-fold lower than in leaf tissue (Fig. 3c). In T-tobacco protoplasts the content of ASA remained constant during 8 d of culture and was greater than the content of DHA (Fig. 3a). However, in NT-tobacco and grapevine protoplasts the content of DHA increased during culture (Fig. 3, b and c). In grapevine protoplasts, the content of DHA was severalfold greater than in NT-tobacco protoplasts. The redox state of ascorbate, expressed as the ratio of ASA to total ascorbate, was higher in both populations of tobacco protoplasts than in grapevine protoplasts, and followed a fairly similar pattern during

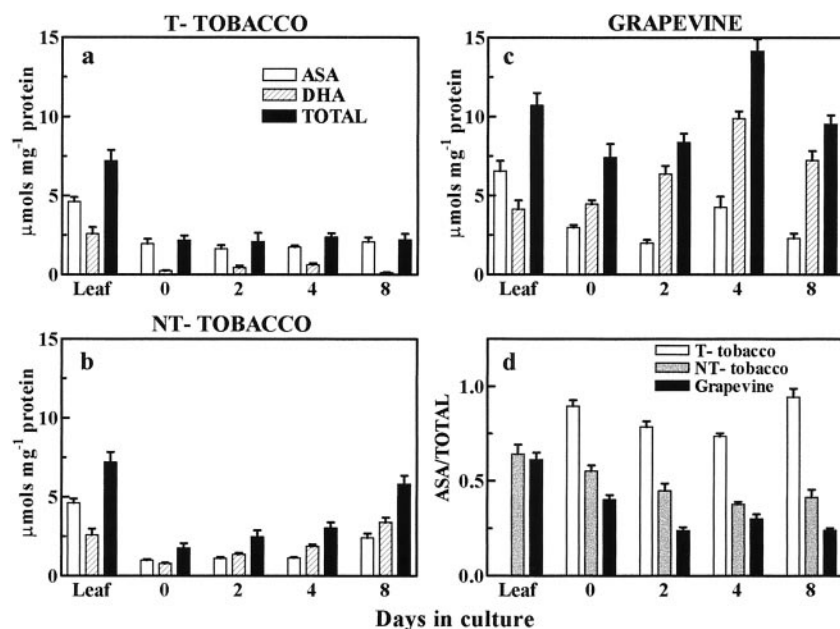
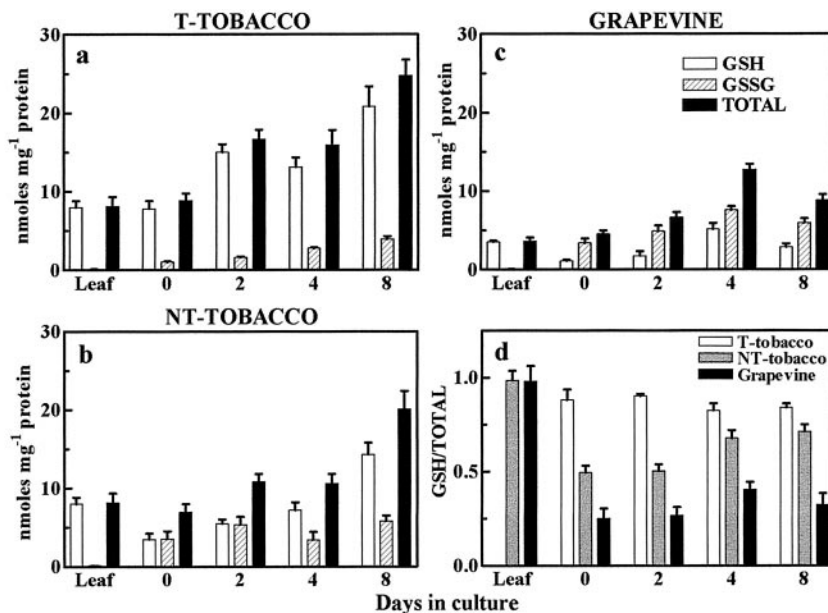


Figure 3. Total intracellular content of ascorbate and its reduced (ascorbate) and oxidized (DHA) forms and the ascorbate redox state (ascorbate/ASA+DHA) in leaf tissue and during protoplast culture. T- and NT- designates totipotent and non-totipotent tobacco protoplasts. Values are the means \pm SE of three independent experiments.

Figure 4. Total intracellular content of glutathione and its reduced (GSH) and oxidized (GSSG) forms and the glutathione redox state (GSH/GSH+GSSG) in leaf tissue and during protoplasts culture. T- and NT- designates totipotent and non-totipotent tobacco protoplasts. Values are the means \pm SE of three independent experiments.



the 8-d culture period in all populations (Fig. 3d); however, the highest redox state was characteristic of the T-tobacco protoplasts.

Tobacco leaf tissue had a 3-fold higher total glutathione content than grapevine leaf tissue; in both cases, glutathione was almost entirely in the reduced form (GSH; Fig. 4). In the two populations of tobacco protoplasts, GSH increased significantly during the 8-d culture period (Fig. 4a); this increase was more pronounced in T- than in NT-tobacco protoplasts (Fig. 4b). In both, the ratio of GSH to total glutathione was greater than 0.5 (Fig. 3d). In contrast, in grapevine protoplasts, the content of GSSG was always greater than that of GSH (Fig. 3c).

Lipid Peroxidation in Cultured Protoplasts

In previous work, we showed that increased lipid peroxidation was accompanied by reduced morphogenic potential (Benson and Roubelakis-Angelakis, 1992, 1994). Fluorescent peroxidized lipids were extracted from leaf tissue and protoplasts, and their concentration was determined by recording their fluorescence intensity and spectra. Fluorescent peroxidized lipids were present in the leaf tissues of both plant species in similar amounts (Fig. 5). In T-tobacco protoplasts, the amounts of fluorescent peroxidized lipids per milligram protein remained fairly constant during culturing, whereas in NT-tobacco protoplasts, it increased 2- to 3-fold during the 8-d period. In grapevine protoplasts, extensive lipid peroxidation was detected and the content of fluorescent peroxidized lipids per milligram protein was almost 2-fold greater than in NT-tobacco protoplasts and more than 3-fold greater than in T-tobacco protoplasts.

SOD, APO, MDHAR, DHAR, GR, and GS-POX Activity in Protoplasts

Various stress conditions are known to induce expression of SOD (Bowler et al., 1992). In freshly isolated T-tobacco protoplasts, SOD activity was slightly higher than in tobacco leaf tissue and continued to increase during the 8-d culture period (Fig. 6). In freshly isolated NT-tobacco protoplasts, SOD activity was more than 2-fold higher than in intact leaf and continued to increase up to d 4 but declined thereafter. The correlation of SOD activity with the presence of O₂⁻ was shown by the addition of alkaline DMSO to the maceration medium for T-tobacco protoplast isolation. The changes in SOD activity during the first 4-d culture paralleled those in NT-tobacco protoplasts (Fig. 6, insert). In grapevine protoplasts, the increase in SOD activity was very low during the entire culture period.

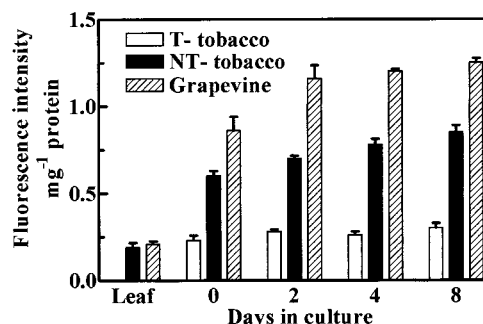


Figure 5. Degree of lipid peroxidation, measured as the accumulation of lipofuscin-like fluorescent compounds in leaf tissue and during protoplasts culture. T- and NT- designates totipotent and non-totipotent tobacco protoplasts. Values are means \pm SE of three independent experiments.

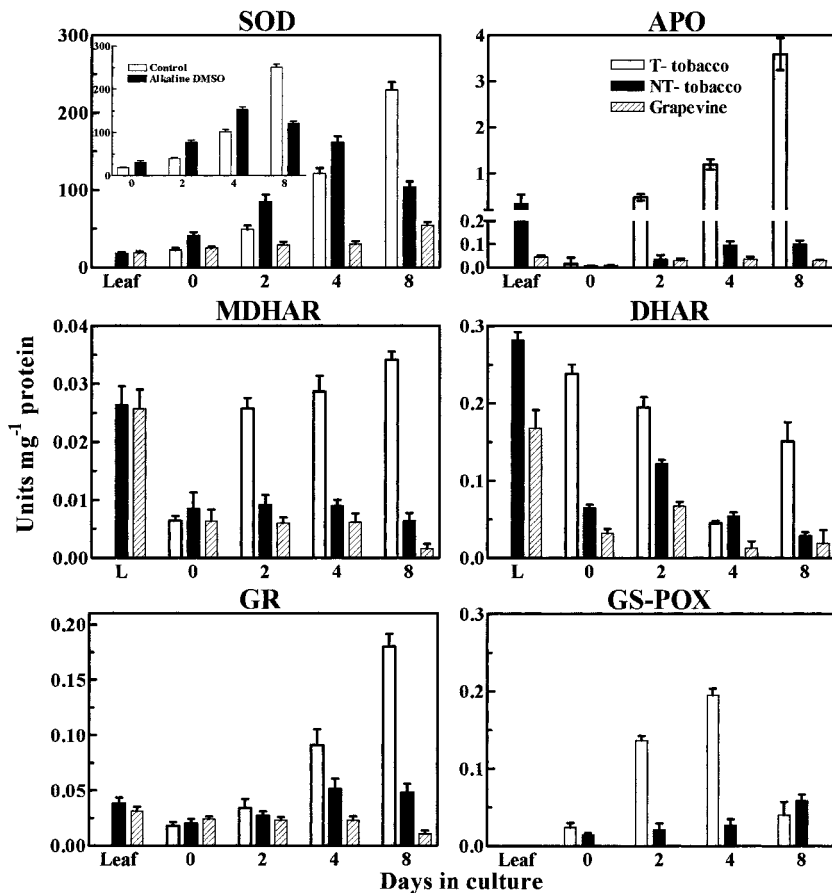


Figure 6. Specific activity of SOD, APO, MDAR, DHAR, GR, and GS-POX during protoplast culture. Insert indicates SOD activity during alkaline DMSO treatment. T- and NT- designates totipotent and non-totipotent tobacco protoplasts. Values are means \pm SE of five independent experiments.

The contribution of individual SOD isoenzymes to total SOD activity was determined by performing SOD assays directly on protein extracts separated in non-denaturing gels. Three and four bands of SOD activity were detected in samples from tobacco and grapevine leaf tissue, respectively (data not shown). In a previous study (Siminis et al., 1994), we have shown that the upper band, in both plant species, represents a mitochondrial MnSOD. The band with higher mobility represents the cytosolic Cu/ZnSOD isoenzyme in both grapevine and tobacco leaf cells. In tobacco the lowest band represents a chloroplastic FeSOD and in grapevine the two bands of the greater mobility contained the chloroplastic Cu/ZnSOD isoenzymes. Leaf extracts from both plant species contained approximately equal amounts of SOD activity (Fig. 6). In tobacco protoplasts, the content of cytoplasmic SOD isoenzyme increased during the early stages of culture and was particularly rapid in NT-tobacco protoplasts (Fig. 7A). This increase was associated with an increase in steady-state levels of Cu/ZnSOD mRNA (Fig. 7B). During the later stages of culture, cytoplasmic SOD remained high in T-tobacco protoplasts but decreased gradually in NT-tobacco protoplasts. In grapevine protoplasts, no significant increase in cytoplasmic SOD content was observed during culture (Fig. 7A). Levels of mitochondrial SOD activity were increased during culture

of protoplasts of both plant species but this induction seems to be independent of the maceration treatments (Fig. 7A). Chloroplastic SOD isoenzymes decreased during culture in all populations of protoplasts. No increase in the content of cytoplasmic SOD was observed up to 6 d after wounding in leaf tissue from both plant species (data not shown). A minor increase was found 8 d after wounding in tobacco, but it was not observed in grapevine.

APO specific activity was severalfold higher in tobacco leaf tissue compared with grapevine leaf tissue (Fig. 6). It was relatively lower in freshly isolated protoplasts but steadily increased in T-tobacco protoplasts during culture, up to a 225-fold increase in activity by d 8. In NT-tobacco protoplasts, APO activity increased up to d 4 in culture and remained constant thereafter, with final levels remaining lower than those in leaf tissue. In grapevine protoplasts, APO activity increased slightly up to d 4 in culture and did not change thereafter. MDAR activity increased only in T-tobacco protoplasts during culturing (Fig. 6). The activity of DHAR, which regenerates the reduced ascorbate pool, was reduced in all populations of protoplasts (Fig. 6). However, the specific activity of DHAR was always severalfold higher in T-tobacco than in NT-tobacco and grapevine protoplasts. In contrast, GR specific activity increased significantly in T-tobacco protoplasts during culturing,

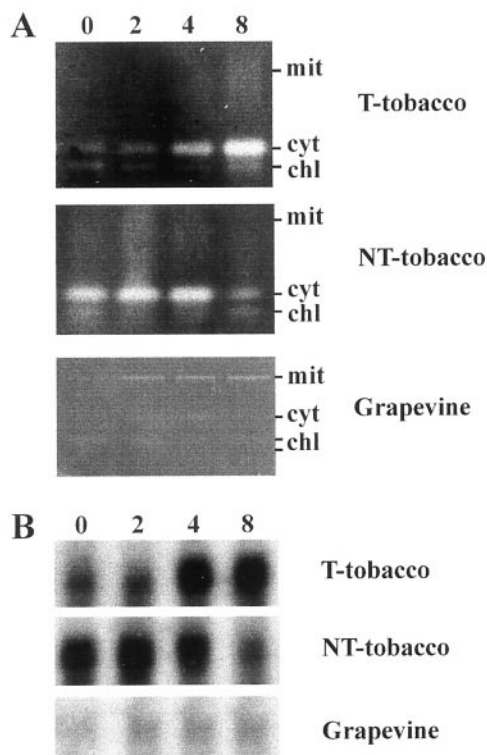


Figure 7. Levels of individual SOD isoenzymes and CuZnSOD mRNA during protoplast culture. A, SOD isoenzyme activity during protoplast culture. Isoenzymes were detected by activity staining of protein extracts separated in non-denaturing gels. Arrows indicate the SOD isoenzymes. mit, Mitochondrial isoenzyme; cyt, cytoplasmic isoenzyme; chl, chloroplastic isoenzyme. B, Steady-state levels of cytoplasmic Cu/ZnSOD mRNA during protoplast culture. T- and NT- designates totipotent and non-totipotent tobacco protoplasts.

as did GS-POX activity, up to d 4 (Fig. 6). The activity of both enzymes was lower in NT tobacco protoplasts compared with T-tobacco protoplasts. In grapevine protoplasts, GR specific activity decreased during culturing and no GS-POX activity could be detected.

DISCUSSION

Increased $O_2^{\cdot -}$ and H_2O_2 accumulation during isolation and culture was accompanied by a decrease in viability and plating efficiency of tobacco protoplasts (Table I, Fig. 1) and a direct correlation between increased AOS and repressed expression of totipotency was demonstrated. This was reinforced by the fact that addition of alkaline DMSO to the maceration medium, normally used to produce T-tobacco protoplasts, resulted in increased content of AOS and production of NT-tobacco protoplasts (Fig. 1). Furthermore, ascorbate increased the viability and plating efficiency of NT-tobacco protoplasts (Fig. 1), in agreement with the results of Ishii (1988), who improved the plating efficiency of rice (*Oryza sativa*) protoplasts by adding SOD and catalase in the maceration medium or GSH in the culture medium. Also, in cultured protoplasts of both tobacco and grapevine,

$O_2^{\cdot -}$ decreased with culture, concomitant with an increase in Mn superoxide dismutase activity, as well as an increase in Cu/Zn SOD activity, although the latter was found only in tobacco protoplasts (Figs. 2 and 7). In NT-tobacco protoplasts, Cu/Zn SOD activity and transcript level decreased by d 8 of culture (Fig. 7) and was accompanied by an increase in intracellular $O_2^{\cdot -}$ (Fig. 2), whereas in T-tobacco protoplasts, Cu/Zn SOD continued to increase throughout the culture period, concomitant with the decrease in the respective intracellular $O_2^{\cdot -}$ (Figs. 2 and 7). Cytosolic Cu/Zn SOD activity (Matters and Scandalios, 1987) and mRNA levels (Van Camp et al., 1994; Willekens et al., 1994) have previously also been shown to increase in maize (*Zea mays*) during stress.

The cellular machinery for H_2O_2 scavenging is very complex; H_2O_2 is removed by catalase in microbodies and mitochondria (Scandalios et al., 1980) and by different isoforms of APO in chloroplasts, cytoplasm, peroxisomes, and mitochondria (Chen and Asada, 1989; Mittler and Zilinskas, 1991; De Leonardis et al., 1995; Yamaguchi et al., 1995). Catalase protective activity has been shown to rapidly increase in tobacco but not in grapevine leaf during maceration with cell wall-degrading enzymes (Siminis et al., 1994), when H_2O_2 starts to accumulate (Papadakis and Roubelakis-Angelakis, 1999). The level of H_2O_2 decreased during protoplast culturing both intra- and extracellularly, except in NT-tobacco and grapevine protoplasts in which the intracellular and extracellular H_2O_2 , respectively, increased by d 8 (Fig. 2). APO activity in cultured T-tobacco protoplasts was strongly enhanced (Fig. 6) and possibly compensated for the decreasing activity of catalase in H_2O_2 scavenging (Siminis et al., 1994). Steady-state levels of cytoplasmic APO mRNA were relatively high in T-tobacco protoplasts, but were not detectable in NT-tobacco protoplasts during culturing (de Marco and Roubelakis-Angelakis, 1996b); therefore, the small increase of APO activity observed in NT-tobacco protoplasts (Fig. 6) could be due to the increased levels of other isoform(s). In NT-tobacco protoplasts, where a significant decrease in catalase activity was observed (Siminis et al., 1994), APO activity increased slightly up to d 4 in culture and remained constant to d 8 (Fig. 6), when the intracellular H_2O_2 content was increased (Fig. 2). In grapevine protoplasts, high catalase but low APO activity was observed (Siminis et al., 1994; Fig. 6), associated with decreased intracellular H_2O_2 (Fig. 2). These results suggest that T- and NT-protoplasts of tobacco and grapevine tissues employ different mechanisms for AOS scavenging.

APO activity depends on the availability of reduced ascorbate produced by the ascorbate-glutathione cycle (Foyer and Halliwell, 1976). MDHAR activity, which regenerates reduced ascorbate, constantly increased during T-tobacco protoplast culturing in parallel to APO activity, whereas DHAR activity decreased (Fig. 6). Reduced ASA slightly decreased in these proto-

plasts during culturing whereas the DHA content remained fairly constant (Fig. 3a). Both ASA and DHA increased significantly in NT-tobacco and grapevine protoplasts but DHA content was always greater than that of reduced ASA (Fig. 3, b and c). The lower redox state of ascorbate in NT-tobacco protoplasts coincided with lower activities of DHAR throughout culture (Figs. 3d and 6).

Glutathione is another component of the Halliwell-Asada pathway, whose reduction is catalyzed by GR. In T-tobacco protoplasts, GR activity increased more than 8-fold by d 8 of culture and coincided with a high content of GSH (Figs. 4a and 6). In contrast, NT-tobacco and grapevine protoplasts exhibited significantly lower GR activity, together with lower GSH levels (Figs. 4, b and c, and 6); in fact, grapevine protoplasts exhibited lower GSH levels compared with GSSG levels during the entire culture period (Fig. 4c). Increased GSH was strongly correlated with protection to oxidative stress in *Arabidopsis* suspension cultures (May and Leaver, 1993), whereas GSSG can be harmful to cells because it can inhibit protein synthesis and enzyme activities (Alscher et al., 1997). The ratio of reduced to oxidized ascorbate and glutathione declined during ozone fumigation and chilling stress (Luwe et al., 1993; Walker and McKersie, 1993). Recent data have shown that changes in the redox status of the cell are among the events that launch the reciprocal up-regulation of defense mechanisms with down-regulation of the cell cycle; increased levels of ASA and/or GSH may be necessary for the G1/S transition (May et al., 1998).

GSH is, in turn, a substrate for GS-POX, the activity of which was below the detection limit in intact leaves of both plant species (Fig. 6). GS-POX activity increased significantly in T-tobacco protoplasts up to d 4 of culture and then decreased; in NT-tobacco protoplasts, GS-POX activity was severalfold lower than in T-tobacco protoplasts and in grapevine protoplasts it was undetectable (Fig. 6). Changes in the redox status of glutathione have been reported to have a regulatory impact on gene expression of cytosolic and chloroplastic isoforms of Cu/Zn SOD (Wingsle and Karpinski, 1996) and of other enzymic AOS scavengers (Karpinski et al., 1997). In grapevine protoplasts with a low redox status of glutathione, cytosolic SOD was not expressed (Figs. 4 and 6). Tepperman and Dunsmuir (1990) suggested that SOD activity is not the limiting factor for cell survival during stress because overexpression of Cu/Zn SOD did not protect plants against paraquat treatment. It seems that crucial is not only the absolute concentrations of AOS, but also their coincidence in the same cell compartment; both are substrates for the Haber-Weiss reaction that produces the very toxic hydroxyl radicals (Bowler et al., 1992). The generation of $\cdot\text{OH}$ can be avoided by a balanced interaction of the compartment-specific isoenzymes of the antioxidant enzymes. In grapevine protoplasts, the activities of

the antioxidant enzymes tested were moderate and declined during culturing (Fig. 6). The loss of effective defense against oxidative stress in both NT-tobacco and grapevine protoplasts is further supported by the detection of high levels of fluorescent lipid peroxides (Fig. 5).

The data presented here provide further evidence for the importance of the antioxidant machinery in plant protoplasts. T- and NT-protoplasts differ in their AOS detoxifying metabolism. Several new questions can now be addressed. Is the high H_2O_2 in NT-tobacco protoplasts responsible for blocking the regenerating potential, and does the low extracellular H_2O_2 level in NT protoplasts prevent the required cell wall reconstitution before the protoplast are able to divide? Why is it that catalase, which has a high K_m for H_2O_2 , does not appear to be capable of effectively scavenging this H_2O_2 ? Why is it that APO, a complementary H_2O_2 scavenger, GR, and GS-POX are not expressed in NT protoplasts, and what is the relationship between the low redox state of ascorbate and glutathione and these deficiencies? Attempts to answer these questions by using molecular approaches may elucidate whether in fact the activity of the antioxidative machinery controls expression of totipotency or whether this is the result of other mechanism(s) that can block the developmental process.

MATERIALS AND METHODS

Plant Material

Protoplasts were isolated from fully expanded, but not senescent, leaves of in vitro-grown grapevine (*Vitis vinifera* L. cv Sultanina; Roubelakis-Angelakis and Zivanovitch, 1991) and greenhouse-grown tobacco (*Nicotiana tabacum* L. cv Xanthi) plants. Small leaf segments (2 mm) were used in all experiments.

Protoplast Experiments

Grapevine and tobacco mesophyll protoplasts were isolated and cultured as already described (Koop and Schweiger, 1985; Katsirdakis and Roubelakis-Angelakis, 1992). The cell wall-hydrolyzing enzymic preparations used in this study were Cellulase R-10, Macerozyme R-10 Onozuka, and purified Cellulase Worthington. Cellulase Onozuka is rich in xylanase, whereas Macerozyme Onozuka and Cellulase Worthington are free of xylanase activity (Fuchs et al., 1989). The macerating enzyme solutions were 1% (w/v) Cellulase R-10, 0.5% (w/v) Macerozyme R-10, or 0.25% (w/v) Cellulase Worthington, 0.5% (w/v) Macerozyme R-10; both exhibited the same cellulase activity. The incubation period with cell wall-hydrolyzing enzymes for isolation of T- and NT- tobacco protoplasts was 4 and 16 h, respectively and of grapevine protoplasts it was 4 h (Siminis et al., 1994). All treatments were in the dark at 25°C.

Viability Staining

Protoplasts were incubated with a 0.01% (w/v) solution of Evans blue in culture medium for 10 min, after which viable protoplasts were identified on the basis of stain exclusion (Graff and Okong O-Ogola, 1971).

Chemiluminescence Assay for $O_2^{\cdot-}$ and H_2O_2

The production of $O_2^{\cdot-}$ and H_2O_2 from protoplasts was determined by a chemiluminescence assay with luminol and lucigenin as substrates, respectively (Murphy and Auh, 1996; Papadakis and Roubelakis-Angelakis, 1999).

Protein Extraction, Enzyme Assays, and Electrophoresis

Total proteins were extracted from leaf tissue and protoplasts as already described (Siminis et al., 1994). In brief, extraction buffer consisted of 0.2 M Tris-HCl (pH 8.0), 5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μ M leupeptin, 10% (w/v) glycerol, 0.25% (w/v) Triton X-100, and 20% (w/v) insoluble polyvinylpyrrolidone. Ascorbate (1 mM) was included when the protein extract was to be used for APO assay. The samples were homogenized with extraction buffer using a Polytron (Ultra Turax T25, probe S15 N 10G) at a speed of 20,000 rpm. The homogenates were centrifuged at 40,000g for 30 min and the supernatants divided into aliquots and frozen at -80°C . The entire extraction procedure was performed at 4°C . Protein determination was performed according to Lowry et al. (1951).

Total SOD (EC 1.15.1.1) activity was determined in crude protein extracts using the photochemical assay developed by Misra and Fridovich (1977). SOD isoenzymes were localized in non-denaturing polyacrylamide gels using the in situ staining technique of Beauchamp and Fridovich (1971). Samples containing 150 μ g protein were electrophoresed on 7.5% (w/v) polyacrylamide gels of 1 mm thickness in a mini-Protein II gel system (Bio-Rad, Hercules, CA), using the Laemmli (1970) buffer system.

APO (EC 1.11.1.11), MDHAR (EC 1.6.5.4), DHAR (EC 1.8.5.1), GR (EC 1.6.4.2), and GS-POX (EC 1.11.1.9) activities were measured as summarized by de Marco and Roubelakis-Angelakis (1996b, 1999). All enzyme assays were carried out at 25°C . Enzyme activities are expressed in units per milligram protein. One unit of activity is defined as the amount of enzyme that oxidizes 1 μ mol of substrate per minute under standard conditions.

RNA Extraction and Blotting

Extraction of RNA from protoplasts was as described by Slater (1988). Total RNA was quantified by spectroscopy and further confirmed by gel electrophoresis and ethidium bromide staining. For RNA-blot analysis, 15 μ g of total RNA was denaturated in formaldehyde, electrophoresed, and transferred to GeneScreen membranes (NEN, Boston) by capillary blotting, according to Sambrook et al. (1989). RNA was fixed on the membranes using 30-s UV exposure followed by 2 h incubation at 90°C . Membranes were hy-

bridized at 60°C with a specific ^{32}P -labeled probe, prepared with random priming (Feinberg and Vogelstein, 1983), using as template a cDNA fragment of 494 bp corresponding to cytoplasmic SOD of *Nicotiana plumbaginifolia* (Tsang et al., 1991).

Photographs of stained gels and northern-blot autoradiographs were scanned with an HP ScanJet 6100 scanner (Hewlett-Packard, Palo Alto, CA) and printed on an HP LaserJet 2100 TN printer using 1,200-dpi photo quality glossy paper, without any visible loss of detail.

Determination of Ascorbate, Glutathione, and Fluorescent Compounds

Total and reduced ascorbate were measured as described by Wang et al. (1991); total and oxidized glutathione were measured according to Akerboom et al. (1981). Lipofuscin-like fluorescent compounds (conjugated Schiff bases) were extracted from leaf tissue and protoplasts with a chloroform-methanol mixture and their concentration was determined by recording their fluorescence spectra and intensity (excitation at 300 and 355 nm, emission at 430 nm). Interfering fluorescent compounds from the lipid extracts (e.g. pigments) were removed with a silica column (Sep Pack, Millipore, Bedford, MA) as has been described earlier (Meir et al., 1992). Fluorescence intensity was expressed as optical density units per milligram protein.

ACKNOWLEDGMENTS

The authors cordially thank Prof. Robin Van Heeswijck (University of Adelaide, Australia) for critically reading this manuscript and Prof. Dirk Inzé (University of Gent, Belgium) for the cDNA encoding cytoplasmic Cu/ZnSOD.

Received July 19, 2000; returned for revision November 4, 2000; accepted February 13, 2001.

LITERATURE CITED

- Akerboom T, Sies H (1981) Assay of glutathione, glutathione disulfide, and glutathione mixed disulfides in biological samples. *Methods Enzymol* **77**: 373–382
- Alscher RG, Donahue J, Cramer CL (1997) Reactive oxygen species and antioxidant: relationships in green cells. *Physiol Plant* **100**: 224–233
- Asada K (1992) Ascorbate peroxidase: a hydrogen peroxide-scavenging enzyme in plants. *Physiol Plant* **85**: 235–241
- Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**: 276–287
- Benson EE, Roubelakis-Angelakis KA (1992) Fluorescent lipid peroxidation products and antioxidant enzymes in tissue cultures of *Vitis vinifera* L. *Plant Sci* **84**: 83–90
- Benson EE, Roubelakis-Angelakis KA (1994) Oxidative stress in recalcitrant tissue cultures of grapevine. *Free Rad Biol Med* **16**: 355–362

- Bowler C, van Montagu M, Inzé D** (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* **43**: 83–116
- Chen G-H, Asada K** (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and their differences in enzymatic and molecular properties. *Plant Cell Physiol* **30**: 987–998
- Cutler A, Saleem M, Wang H** (1991) Cereal protoplast recalcitrance. *In Vitro Cell Dev Biol* **27**: 104–111
- De Leonardis S, De Lorenzo G, Borraccino G, Dipierro S** (1995) A specific ascorbate free radical reductase isoenzyme participates in the regeneration of ascorbate for scavenging toxic oxygen species in potato tuber mitochondria. *Plant Physiol* **109**: 847–851
- de Marco A, Roubelakis-Angelakis KA** (1996a) The complexity of enzymic control of hydrogen peroxide concentration may affect the regeneration potential of plant protoplasts. *Plant Physiol* **110**: 137–145
- de Marco A, Roubelakis-Angelakis KA** (1996b) Hydrogen peroxide plays a bivalent role in the regeneration of protoplasts. *J Plant Physiol* **149**: 109–114
- de Marco A, Roubelakis-Angelakis KA** (1997) Laccase activity could contribute to cell-wall reconstitution in regenerated protoplasts. *Phytochemistry* **46**: 421–425
- de Marco A, Roubelakis-Angelakis KA** (1999) Specific features of the ascorbate/glutathione cycle in cultured protoplasts. *Plant Cell Rep* **18**: 406–411
- Eshdat Y, Holland D, Faltin Z, Ben-Hayyim G** (1997) Plant glutathione peroxidases. *Physiol Plant* **100**: 234–240
- Feinberg AP, Vogelstein B** (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specificity. *Anal Biochem* **132**: 6–13
- Foyer CH, Descourvieres P, Kunert KJ** (1994) Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. *Plant Cell Environ* **17**: 507–523
- Foyer CH, Halliwell B** (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* **133**: 21–25
- Foyer CH, Lopez-Delgado H, Dat JE, Scott IM** (1997) Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. *Physiol Plant* **100**: 241–254
- Fuchs Y, Saxena YA, Gamble HR, Anderson JD** (1989) Ethylene biosynthesis-inducing protein from cellulisin is an endoxylanase. *Plant Physiol* **89**: 138–143
- Graff DF, Okong O-Ogola O** (1971) The use of non-permeating pigments for testing the survival of cells. *J Exp Bot* **22**: 756–758
- Hyland K, Voisin E, Banoun H, Auclair C** (1983) Superoxide dismutase assay using alkaline dimethylsulfoxide as superoxide anion-generating system. *Anal Biochem* **135**: 280–287
- Iiyama K, Lam TB-T, Stone BA** (1994) Covalent cross-links in the cell wall. *Plant Physiol* **104**: 315–320
- Ishii S** (1987) Generation of active oxygen species during enzymic isolation of protoplasts from oat leaves. *In Vitro Cell Dev Biol* **23**: 653–658
- Ishii S** (1988) Factors influencing protoplast viability of suspension-cultured rice cells during isolation process. *Plant Physiol* **88**: 26–29
- Jabs T, Tschope M, Colling C, Hahlbrock K, Scheel D** (1997) Elicitor-stimulated ion fluxes and superoxide from the oxidative burst are essential components in triggering defense gene activation and phytoalexin synthesis in parsley. *Proc Natl Acad Sci USA* **94**: 4800–4805
- Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux P** (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in *Arabidopsis* during excess light stress. *Plant Cell* **9**: 627–640
- Katsirdakis KC, Roubelakis-Angelakis KA** (1992) A modified culture medium and culture conditions increase viability and cell wall synthesis in grapevine (*Vitis vinifera* L. cv Sultanina) leaf protoplasts. *Plant Cell Tissue Organ Cult* **28**: 255–260
- Koop HV, Schweiger HG** (1985) Regeneration of plants from individually cultivated protoplasts using an improved microculture system. *J Plant Physiol* **121**: 245–257
- Laemmli UK** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685
- Lamb C, Dixon R** (1997) The oxidative burst in plant disease resistance. *Annu Rev Plant Physiol Plant Mol Biol* **48**: 251–275
- Larson RA** (1988) The antioxidants of higher plants. *Phytochemistry* **27**: 969–978
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275
- Luwe MF, Takahama U, Heber U** (1993) Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves. *Plant Physiol* **101**: 969–976
- Matters GL, Scandalios JG** (1987) Synthesis of isozymes of superoxide dismutase in maize leaves in response to O₃, SO₂ and elevated O₂. *J Exp Bot* **38**: 842–852
- May MJ, Leaver CJ** (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiol* **103**: 621–627
- May MJ, Vernoux T, Leaver C, Van Montagu M, Inzé D** (1998) Glutathione homeostasis in plants: implications for environmental sensing and plant development. *J Exp Bot* **49**: 649–667
- Meir S, Philosoph-Hadas S, Aharoni N** (1992) Ethylene-increased accumulation of fluorescent lipid-peroxidation products detected during senescence of parsley by a newly developed method. *J Am Soc Hortic Sci* **117**: 128–132
- Misra HP, Fridovich I** (1977) Superoxide dismutase: a photochemical augmentation assay. *Arch Biochem Biophys* **181**: 308–312
- Mittler R, Zilinskas BA** (1991) Purification and characterization of pea cytosolic ascorbate peroxidase. *Plant Physiol* **97**: 962–968
- Murphy TM, Auh CM** (1996) The superoxide synthases of plasma membrane preparations from cultured rose cells. *Plant Physiol* **110**: 621–629

- Noctor G, Foyer CH** (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 249–79
- Papadakis AK, Roubelakis-Angelakis KA** (1999) The generation of active oxygen species differs in *Nicotiana* and *Vitis* plant protoplasts. *Plant Physiol* **121**: 197–205
- Roubelakis-Angelakis KA** (1993) An assessment of possible factors contributing to recalcitrance of plant protoplasts. In KA Roubelakis-Angelakis, K Tran Thanh Van, eds, *Morphogenesis in Plants: Molecular Approaches*. Plenum, New York, pp 201–220
- Roubelakis-Angelakis KA, Zivanovitch S** (1991) A new culture medium for in vitro rhizogenesis of grapevine (*Vitis* spp.) genotypes. *Hortscience* **26**: 1552–1555
- Sambrook J, Fritsch EF, Maniatis T** (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Scandalios JG** (1993) Oxygen stress and superoxide dismutases. *Plant Physiol* **101**: 7–12
- Scandalios JG, Tong W-F, Roupakias DG** (1980) Cat 3, a third gene locus coding for tissue-specific catalase in maize: genetics, intracellular localization, and some biochemical properties. *Mol Genet* **179**: 3–41
- Siminis CI, Kanellis AK, Roubelakis-Angelakis KA** (1993) Differences in protein synthesis and peroxidase isoenzymes between recalcitrant and regenerating protoplasts. *Plant Physiol* **87**: 263–270
- Siminis CI, Kanellis AK, Roubelakis-Angelakis KA** (1994) Catalase is differentially expressed in dividing and nondividing protoplasts. *Plant Physiol* **105**: 1375–1383
- Slater A** (1988) Extraction of RNA from plants. In JM Walker, ed, *New Nucleic Acids Techniques*. The Humana Press, Totowa, NJ, pp 437–446
- Tepperman JM, Dunsmuir P** (1990) Transformed plants with elevated levels of chloroplastic SOD are not more resistant to superoxide toxicity. *Plant Mol Biol* **14**: 501–511
- Tsang EWT, Bowler C, Herouart D, Van Camp W, Villarroel R, Genetello C, Van Montagu M, Inzé D** (1991) Differential regulation of superoxide dismutases in plants exposed to environmental stress. *Plant Cell* **3**: 783–792
- Van Camp W, Willekens H, Bowler C, Van Montagu M, Inzé D, Reupold-Popp P, Sandermann H, Langebartels C** (1994) Elevated levels of SOD protect transgenic plants against ozone damage. *Biotech* **12**: 165–168
- Walker MA, McKersie BD** (1993) Role of the ascorbate-glutathione system in chilling resistance of tomato. *J Plant Physiol* **141**: 234–239
- Wang SY, Jiao HJ, Faust M** (1991) Changes in ascorbate, glutathione, and related enzyme activities during thiadiazuron-induced bud break of apple. *Physiol Plant* **82**: 231–236
- Willekens H, Van Camp W, Van Montagu M, Inzé D, Langebartels C, Sandermann H** (1994) Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. *Plant Physiol* **106**: 1007–1014
- Wingsle G, Karpinski S** (1996) Differential redox regulation by glutathione of glutathione reductase and CuZn-superoxide dismutase gene expression in *Pinus sylvestris* L. needles. *Planta* **198**: 151–157
- Yamaguchi K, Mori H, Nishimura M** (1995) A novel isoenzyme of ascorbate peroxidase localized on glyoxysomal and leaf peroxisomal membranes in pumpkin. *Plant Cell Physiol* **36**: 1157–1162